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A Collaborative Translational Autism Research Program for the Military

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for September 2012 to December 2015

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14. ABSTRACT

The central hypothesis for the proposed research is that a collaborative translational autism research program with the military will result in the improved diagnosis and care of those enrolled in the study and enhance biomedical research on the diagnosis, causes, and treatment of autism spectrum disorders (ASD), in general. With approved revised Specific Aims, during this project, we enrolled 65 families (259 individuals) in the existing Central Ohio Registry for Autism, including 26 families from Wright-Patterson Air Force Base. Ninety-six percent of these families had a chromosomal microarray analysis performed. Exome sequencing was performed on 210 individuals from Autism I and II with the identification of 7 clinically relevant likely pathogenic variants in probands related to their ASD diagnosis. Transcriptome analysis and subsequent analysis in Central Ohio Registry for Autism trios of 14 single nucleotide polymorphisms in relevant genes found a significant association between a variant in the serotonin receptor 2A gene that modulates its expression. Preliminary data also suggest an association between ASD and the immune-related genes *C4A* and *DEFA3*. Finally, using a mouse model for an X-linked gene involved in cholesterol synthesis, we demonstrated behavioral abnormalities consistent with a role for the pathway in neural development and, perhaps, ASD.

15. SUBJECT TERMS
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TABLE OF CONTENTS

Section **Page**

LIST OF FIGURES

Page

LIST OF TABLES

Page

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1.0 SUMMARY

The central hypothesis for the proposed research is that a collaborative translational autism research program with the military will result in the improved diagnosis and care of those enrolled in the study and enhance biomedical research on the diagnosis, causes, and treatment of autism spectrum disorders (ASD), in general. With approved revised Specific Aims, during this project, we enrolled 65 families (259 individuals) in the existing Central Ohio Registry for Autism, including 26 families from Wright-Patterson Air Force Base. Ninety-six percent of these families had a chromosomal microarray analysis performed. Exome sequencing was performed on 210 individuals from Autism I and II with the identification of 7 clinically relevant likely pathogenic variants in probands related to their ASD diagnosis. Transcriptome analysis and subsequent analysis in Central Ohio Registry for Autism trios of 14 single nucleotide polymorphisms in relevant genes found a significant association between a variant in the serotonin receptor 2A gene that modulates its expression. Preliminary data also suggest an association between ASD and the immune-related genes *C4A* and *DEFA3*. Finally, using a mouse model for an X-linked gene involved in cholesterol synthesis, we demonstrated behavioral abnormalities consistent with a role for the pathway in neural development and, perhaps, ASD.

2.0 INTRODUCTION AND BACKGROUND

Autism spectrum disorder (ASD) is the fastest growing developmental disability in the United States, costing more than $90 billion/year, and is, therefore, extremely important to the Department of Defense (DoD). According to the *Military Times*, "autism is the most prevalent special-needs issue in military families" [1]. ASDs have a frequency of ~1/68 according to the National Health Interview Survey of the Centers for Disease Control and Prevention [2], with a similar incidence in the military. Further, it is estimated that genetic factors account for up to 70-80% of the risk for an ASD [3,4]. Chromosome microarray analysis (CMA) has replaced a standard karyotype as a first tier test; it offers a diagnostic yield of 15-20% and detects large as well as submicroscopic copy number variations (CNVs) in the form of deletions and duplications that contribute or are occasionally causative of disease [5]. More recently, the importance of rare inherited and *de novo* non-synonymous missense, nonsense, and splice site single nucleotide variants has emerged as a result of advances in whole-exome sequencing (WES) of large research cohorts of ASD families [6-9]. A unifying theme among many of the CNVs identified, as well as susceptibility loci ascertained by linkage, association, and direct gene sequencing studies, is localization of the involved protein at neural synapses. While the availability of CMA has greatly improved the clinical diagnosis rates, 70-75% of children with an ASD are still without an identifiable genetic etiology. In these cases, the ASD likely results from a combination of effects of multiple genes and environmental factors (multifactorial inheritance). Understanding the underlying causes of ASDs may help direct current and future medical management and behavioral and physical therapies, predict future outcomes for the child, and provide recurrence risk estimates for the immediate and extended family.

The Exceptional Family Member Program initiative has resulted in improved services for military families; however, the complexities of ASD diagnoses and treatments, and advances in translational research, including genetics, demand continued efforts to improve the quality of care. Specific military preparedness issues for families with a child with an ASD include the

stress of deployments and frequent moves, early retirement or lack of reenlistment for family concerns, and limited community resources at many smaller bases. ASDs pose a particularly high level of stress for many military families; however, substantial improvements are attainable through collaborative translational research linked to better care.

In 2006, the Central Ohio Registry for Autism (CORA) was initiated as a collaboration between Wright Patterson Air Force Base (WPAFB) and Nationwide Children's Hospital (NCH). The primary purpose of CORA was to develop a comprehensive autism registry for genetic and other studies for military and civilian families in central Ohio. Congressionally supported funding for the project was secured, beginning in late 2009 (total $2.77 million, 9/30/09-9/29/12), to support (1) development of the CORA registry, (2) expansion of diagnostic and treatment services for WPAFB families through a collaboration with Dayton Children's Medical Center, (3) molecular studies to identify novel autism susceptibility genes, and (4) cost and satisfaction analyses for the military services components in Aim 2. The timeframe from 9/30/09-9/29/12 is referred to as Autism I, and data and enrollment statistics occurring during this period will be noted as such.

In 2012, with the procurement of an additional 3 years of funding, the project (referred to as Autism II) was continued to (1) further expand CORA to provide military and civilian families with the opportunity to participate in translational research and to foster academic/military collaboration; (2) identify new autism susceptibility variants using next generation sequencing (NGS) technology by (a) direct exome sequencing of selected families in the registry and (b) association studies of novel regulatory and gene splicing variants that demonstrate allelic expression imbalance (AEI) in prefrontal cortex between autism and control brains; and (3) demonstrate the significance of two candidate autism susceptibility genes by performing functional studies using a live mouse model.

Over the course of Autism II, there have been several changes to the Statement of Work as discussed below. Aims and results in this final report have been updated to reflect these changes. Additionally, a no cost extension was approved to continue the cooperative agreement through the end of December 2015 to allow further time for ongoing research studies and to coincide with the end of the fiscal year for Nationwide Children's Hospital and The Ohio State University (OSU). The latter has facilitated the transition of project staff to other positions within the organization.

Aim 4.1 – Due to a delay in obtaining AF Institutional Review Board (IRB) approval, patient enrollment in Autism II did not begin until February 2013. Staffing changes at WPAFB necessitated a suspension of recruitment from June through September 2014 and then a decrease in available recruitment dates to every other month starting in October 2014. An additional challenge to recruitment was identified when it was determined that eligible families were experiencing "research fatigue" when they were being approached for research participation in multiple specialty clinics and sometimes for multiple research studies even within the same clinic appointment. Mrs. Hansen-Kiss reviewed 885 children being seen at NCH Child Development Center or Genetics between 12/1/2013 and 01/31/2015. Of the 196 children determined to be eligible for CORA, 16 enrolled (8%), 72 declined out right (37%), and 111 took information but did not enroll or failed to attend their scheduled clinical appointment. Finally, the original goal of 50 families for Year 3 was revised after it was determined that study enrollment would be closed at the end of April 2015 to allow enough time for participating families to complete necessary blood draws and psychological testing. **These changes resulted in decreased recruitment of families in Autism II as shown below.**

Aim 4.2.1 – In our proposal, we planned to perform exome sequencing on 100 individuals in Year 1, 80 in Year 2, and 30 in Year 3. Final numbers of families sequenced reached the goal of 210 individuals sequenced in Autism II, although more individuals were sequenced in Year 3 and less in Years 1 and 2.

Aim 4.2.4 – Revised association studies included analysis of CNVs involving immune related genes (complement C4 and DEFA defensin genes) in collaboration with Dr. Yu, Nationwide Children's Hospital, in addition to single nucleotide polymorphisms (SNPs) with AEI discovered by Dr. Sadee, OSU subcontract.

Aim 4.3 – Dr. Herman obtained permission in August 2013 to utilize the existing conditional *Nsdhl* mouse model developed in her lab as Mouse Model #1. Additionally, after careful review of the genes identified as potential candidates during WES, it was determined that no suitable gene for a creation of Mouse Model #2 had been identified. The requirement for a second mouse model was removed.

The above modifications and reduced scope of work resulted in a revised budget and extension of the project through 12/31/15. The revisions were approved by DoD 5/26/15.

3.0 AIM 4.1 – EXPAND CORA

To be eligible for enrollment in CORA, families must have a child with a previous diagnosis of an ASD and at least one biological parent willing to participate and must have completed psychological testing that includes the Autism Diagnostic Observation Schedule (ADOS) and genetic testing that includes Fragile X and a genomic oligonucleotide chromosomal microarray. Whenever possible both parents and any siblings were enrolled, and extended family members were enrolled on a limited basis contingent on Dr. Herman's approval.

Since not all families had equal access to psychological and genetic testing options, families who would otherwise be eligible were also considered if the additional testing could be completed by the research study. In particular, the ADOS, which is considered the "gold standard" in research psychological assessment of ASDs, and intelligence quotient (IQ) (Stanford-Binet and Leiter-R) testing were offered to all families that had not had previous testing completed on a clinical basis. As discussed below, selected affecteds also had a CMA performed as part of CORA.

Finally, families were asked to provide a blood sample on all enrolled family members, complete a pregnancy questionnaire, complete a 3-generation family medical history (pedigree), provide consent for release of medical records pertaining to the ASD diagnosis from agencies outside of NCH and the AF, and complete pen-and-paper psychological surveys on parents and unaffected siblings (Social Responsiveness Scale (SRS) Adult and Child versions).

3.1 Task 4.1.1 – Enroll 50 Families (25 NCH/25 WPAFB) in Year 1 and Year 2; Enroll 15 Families (10 NCH/5 WPAFB) in Year 3

Seventy-three families were enrolled during Autism II. Eight families were withdrawn, leaving **65 actively enrolled families in CORA during Autism II** (Table 1), with a total of 259 individuals. Including those individuals who enrolled as part of Autism I, there are 304 actively enrolled families with 1160 individuals (Table 2) in CORA. For the 8 families withdrawn from Autism II (all NCH families), 6 of the families failed to provide a blood sample on the child with autism, and at least 1 parent within 1 year of enrollment after multiple requests and 2 families requested to be withdrawn stating personal conflicts.

Table 1. Autism II Statistics

Statistic	N
Active Individuals Enrolled	259
Active Families Enrolled	65
Recruited from NCH	39 (60%)
Recruited from WPAFB	26 (40%)
Multiplex Families	10
Simplex Families	55
Total Affected Children	76
Male	63
Female	13
Total Microarray Completed	73 (96%)
NCH	46
WPAFB	27
Total ADOS Completed	73 (96%)
NCH	46
WPAFB	27
Total IQ Testing Completed	69 (91%)
NCH	43
WPAFB	26
Withdrawn Families[a]	8
Withdrawn Individuals[a]	30
Total Families Enrolled	73
Total Individuals Enrolled	289

[a]Withdrawn families/individuals are not included in the dataset used to generate the statistics for Table 1. These data reflect ACTIVE families only.

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- 47 families (189 individuals) enrolled as part of Autism II
  - 16 families enrolled during Year 1
  - 22 families enrolled during Year 2
  - 9 families enrolled during Year 3
- 8 families (30 individuals) enrolled, but were later withdrawn

Wright-Patterson AFB

- 26 families (100 individuals) enrolled as part of Autism II, currently active
  - 9 families enrolled during Year 1
  - 10 families enrolled during Year 2
  - 7 families enrolled during Year 3
- No families were withdrawn

Table 2. Combined Autism I and II Statistics

Statistic	N	
Active Subjects Enrolled	1160	
Active Families Enrolled[a]	304	
Recruited from NCH	200 (66%)	
Recruited from WPAFB	104 (34%)	
Multiplex Families	50	
Simplex Families	254	
Total Affected Children	364	
Diagnosis	Male	Female
Autism Disorder	160	30
Autism Spectrum Disorder	46	15
Asperger Syndrome	31	5
PDD-NOS	64	13
Total Microarray Completed	346 (95%)	
NCH	224	
WPAFB	122	
Total ADOS Completed	343 (94%)	
NCH	222	
WPAFB	121	
Total IQ Testing Completed	273 (75%)	
NCH	184	
WPAFB	89	
History of Seizures	27 (8%)	
Macrocephaly	78 (21%)	
Total Complete Trio Families[b]	236 (78%)	
NCH	147	
WPAFB	89	
Total SuperSTAR Families[c]	225 (74%)	
NCH	134	
WPAFB	91	

Note: PDD-NOS = pervasive developmental disorder not otherwise specified.

[a]Withdrawn families/individuals are not included in the dataset used to generate the statistics for Table 2. These data reflect ACTIVE families only. In total 37 families (125 individuals) have been withdrawn.

[b]Complete Trio Families are families where at least 1 child with an ASD and both biological parents are participating and have provided a blood sample.

[c]SuperSTAR Families are families that have completed all requirements for enrollment in CORA; SuperSTAR families may not be Complete Trios.

3.2 Task 4.1.2 – Completion of ADOS on >90% of Individuals with an ASD Diagnosis

3.2.1 ADOS Testing. ADOS testing, both clinical and through the research study, was completed on 73/76 children (96%) with an ASD diagnosis (Table 1). The remaining 3 children (1 family) live greater than 2 hours from Columbus, and the family was unable to arrange for the psychological testing to be completed.

3.2.2 IQ Testing. IQ testing, both clinical and through the research study, was completed on 69/76 (91%) of the children in Autism II (Table 1). Thirty-six of these children (52%) were found to have an IQ less than 80, which is consistent with a diagnosis of intellectual disability. Additionally, 15/69 children (21%) were found to have an IQ less than 55, which falls in the moderate to profound range of intellectual disability. These data are similar to the larger CORA cohort (Table 2) with 123/273 (45%) children with an IQ less than 80 and 46/273 (17%) children with an IQ less than 55 in the moderate to profound range.

3.2.3 Research Microarrays. Twenty-nine CMAs were performed as part of Autism II research in cases where insurance, including TriCare, would not provide reimbursement for a clinical CMA. Military families were given preference for the research arrays since they were previously unable to obtain a clinical CMA due to restrictions in coverage with TriCare insurance. The research arrays were performed in the NCH clinical Molecular Genetics Laboratory using coded numbering and are identical to the arrays that patients receive as a clinical test. All of the research array results were reviewed by Dr. Herman (Table 3), and with IRB approval, summary letters explaining results of the testing were sent to families if current contact information was available. **No likely pathogenic or pathogenic findings on CMA were identified in Autism II.** However, one of the variants of unknown significance (6q26 loss *PARK2*, associated with an autosomal recessive form of Parkinson disease) was identified in a family from WPAFB. Although the result was unrelated to the ASD diagnosis in the child, it was felt to have potential future implications for offspring or other family members. Dr. Boreman and Dr. Schulteis, WPAFB, were notified of the finding, and the family received clinical genetic counseling by Dr. Herman at NCH related to the results.

Table 3. Research Microarray Results

Microarray Results	Total
Normal	17
Benign	4
Likely Benign	0
Variant of Unknown Significance[a]	8
Likely Pathogenic	0
Pathogenic	0
Total	29
	12 NCH and 17 WPAFB

[a]One variant of unknown significance, although unrelated to the ASD diagnosis, was felt to have possible medical implications, and the family was seen for a clinical appointment by Dr. Herman [6q26 loss (*PARK2*, including exon 3)].

3.3 Task 4.1.3 – Prepare DNA and Lymphoblast Cell Lines on All Newly Enrolled Individuals with a Success Rate of Lymphoblast Transformation >90%

Blood obtained as part of the study was separated into two sample types: one from which primary DNA was isolated from whole blood and the other treated to create lymphoblastoid cell lines. The sample types that were collected on participants have changed several times over the life of the CORA study. However, during the majority of Autism II, children with an ASD diagnosis and parents were drawn for both a primary DNA sample and cell lines; enrolled unaffected siblings were drawn for a primary DNA sample only. Blood samples were sent to the NCH Cell Line Core for isolation of the primary DNA and creation of the lymphoblastoid cell lines. Lymphoblastoid cell lines provide a source of unlimited DNA by regrowth of cells. However, the process of cell line creation may introduce new mutations that are not present in the participant. Therefore, primary DNA is preferred for exome sequencing and also allows confirmation of molecular findings identified.

For the 259 active participants enrolled in Autism II, 250 have had their blood drawn. Fifty participants were drawn for primary DNA only (unaffected siblings). Of the 200 participants drawn for cell line preparation, all samples (100%) were successfully transformed. Of the 1,160 participants actively enrolled in CORA (Autism I and II), 1,124 have had their blood drawn. Cell lines were created for 1,033 participants, and primary DNA was isolated for 1,026 participants.

Of the 9 participants in Autism II who failed to have blood drawn after multiple requests, 4 were parents, 4 were siblings, and 1 was an extended family member.

3.4 Task 4.1.4 – Complete Pregnancy Questionnaire, 3-Generation Pedigree, and Enter Phenotypic Data into the Database on >90% of Newly Enrolled Families

3.4.1 Pregnancy Questionnaire (PQ). A PQ was completed for each child with an ASD diagnosis. As part of Autism I, an online version of the PQ was created. However, in 2013 technical difficulties with the online program and the product vendor were identified, and it was determined that a new online survey platform would be required. In the meantime, families were provided with a paper copy of the PQ and asked to complete and return it to the study.

Mrs. Hansen-Kiss worked closely with the NCH Research Information Technology department and in December 2013 it was determined that the RedCap platform would provide the best online survey option. Unfortunately, given the time required to train Mrs. Hansen-Kiss on the RedCap platform and to create and test/validate the survey instrument, it was determined that the online survey would not be available to families until the second half of 2014. With the estimated closure of the CORA study in early 2015, we continued using the paper PQ forms. Historically, completion of the paper version is much lower than the online version and has had a lower completion rate.

Families were provided with multiple reminders by both phone and mail and were also given the option of completing the PQ over the phone with the study psychometrician or study coordinator. Sixty-one (80%) of the children enrolled through Autism II have a completed PQ.

3.4.2 Generation Pedigree. Families completed a 3-generation family medical history (pedigree) either at the time of enrollment or over the phone with the study coordinator. A 3-generation pedigree was completed by Mrs. Hansen-Kiss on 64/65 (98%) of the families enrolled during Autism II.

3.4.3 Entering Phenotypic (and Other) Data. Data gathered from families were entered into the secure database that was created as part of Autism I and utilized SharePoint and Microsoft InfoPath platforms for the front-end interface and SQL Server for back-end data storage. Data were entered for 100% of the enrolled families. Additionally, periodic quality control measures, such as chart audits and data analysis, were completed to ensure integrity of the data entered.

3.4.4 Psychological Surveys on Parents and Unaffected Siblings. Broader autism phenotype (BAP) has been defined as having some traits seen in ASD but not enough to meet the criteria for an ASD diagnosis [10]. Features of the BAP in close relatives of an individual diagnosed with an ASD support multifactorial inheritance of risk and could be employed to help map and define these "risk genes" [11,12]. To assess the BAP in CORA, families were asked to complete the SRS Adult or Child form on parents and unaffected siblings [13]. Completed forms were returned for 33 unaffected siblings in Autism II, and data are available for 96 unaffected siblings for the entire CORA cohort. Completed forms were returned for 88/121 (73%) parents in Autism II. Seventy-seven of these parents (86%) had an SRS score in the normal range (≤59), 5 (6%) had a score in the mild-moderate range (60-75), and 6 (7%) had scores in the severe range (≥76). SRS data are available for 425 parents for the entire CORA cohort.

Dr. Megan Norris, PhD, a licensed psychologist at the NCH Child Development Center and Clinical Assistant Professor of Pediatrics at OSU, recently submitted a request to Dr. Herman to complete secondary analysis on de-identified data from parental and sibling SRS data in CORA. After a careful review of the current literature, Dr. Norris believes the CORA cohort may represent the largest population of parental SRS scores currently available. Dr. Norris is proposing to review the data to explore patterns of scores and correlations between scores in parents and unaffected siblings with those of children with an ASD diagnosis. She hopes to determine if symptom severity in the child with the ASD is predictive of SRS scores in other family members. Dr. Norris is currently preparing the study protocol for NCH IRB review. Dr. Herman and Mrs. Hansen-Kiss will be co-investigators on the study. It is hoped that a future peer-reviewed journal article and a poster presentation at a national conference may result from these data.

3.5 Disposition of CORA Registry at Close of Study

At the end of this cooperative agreement with the DoD, de-identified DNA samples (primary and cell line DNA), aliquots of frozen lymphoblastoid cell lines, and de-identified data will be transferred to the Autism Sequencing Consortium (ASC) led by Dr. Joseph Buxbaum at the Icahn School of Medicine at Mt. Sinai in New York. The ASC was established in 2010 and is a National Institutes of Health funded collaboration of international leaders in the field of autism research. More information on the ASC can be found at https://genome.emory.edu/ASC.

Prior to the transfer of any de-identified DNA samples or de-identified data, a Material Transfer Agreement, Data Use Agreement, and Data Use Limitation Record were put into place between NCH and the ASC. Families were also given the ability to opt out of having their

samples and data transferred to the ASC. The procedures for transfer of samples and de-identified data and ability to opt out of the transfer were reviewed and approved by the NCH IRB (Amendment 00010915 on 11/10/15) as well as the Air Force Medical Support Agency Research Oversight and Compliance Division (SGE-C) (letter of 11/13/15). **To ensure that there is sufficient time to allow families in CORA to opt out, if desired, and then ship samples and transfer data to Dr. Buxbaum, Dr. Herman requested a no cost extension through 2/29/16.** Five families opted out of the transfer to the ASC.

Dr. Herman will be a participating member of the ASC team. As such, should a medically significant finding be identified on a CORA family as part of the ASC research, Dr. Herman will have the ability to go back into the CORA study database at NCH and re-contact families and provide necessary genetic counseling as appropriate related to the results. Dr. Herman expects that she will maintain an active CORA study protocol with the NCH IRB for a period of 3-5 years, or until such time as she is no longer employed by NCH. Once Dr. Herman closes the NCH CORA protocol, the CORA database at NCH will be destroyed and there will be no way to link the de-identified DNA samples and de-identified data back to the families.

3.6 PTEN Study

PTEN (phosphatase and tensin homologue deleted on chromosome ten) is a tumor suppressor gene that functions primarily as a cellular lipid phosphatase [14]. Mutations in the *PTEN* gene have been identified in a group of related syndromes currently referred to as PTEN hamartoma tumor syndromes [15,16]. These include Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, and some cases of Proteus and Proteus-like syndromes. Individuals with Cowden syndrome typically develop multiple benign hamartomas and have an increased risk of certain cancers, particularly of the breast, uterus, and thyroid. Bannayan-Riley-Ruvalcaba syndrome is characterized by macrocephaly, hamartomas (including lipomas, hemangiomas, or intestinal polyps), penile freckling in males, and developmental delay, including an increased risk of developing an ASD.

Over the past ~10 years, work from several groups, including ours, has demonstrated that 5-10% of children with an ASD and macrocephaly have a heterozygous pathogenic *PTEN* mutation as the underlying cause [17-20]. During Autism I, we initiated a separate IRB protocol (Dr. Herman, principal investigator) to collect phenotypic data and DNA on children with a pathogenic *PTEN* mutation and their families. We used exome sequencing to examine additional genes that may act as secondary or modifier loci and influence the developmental and cognitive phenotype. Such genes could include (1) those in the PTEN or related pathways, (2) genes known to be associated with susceptibility to ASD, (3) genes that cause monogenic isolated or syndromic intellectual disability, and (4) genes associated with macrocephaly or brain growth. Genes identified in this fashion would become candidate susceptibility loci for ASD in individuals without a *PTEN* mutation.

The PTEN study (Table 4) ran in parallel to the CORA study. Families from central Ohio identified to have a child with a pathogenic *PTEN* mutation and a diagnosis of ASD enrolled through the CORA study. Families living in Ohio, where the child had a pathogenic *PTEN* mutation but did not have an ASD diagnosis, enrolled through the PTEN study. All *PTEN* positive families from outside of Ohio, with or without a child with an ASD diagnosis, enrolled

through the PTEN study. Molecular studies on *PTEN* families were conducted through the PTEN protocol.

Table 4. PTEN Study Enrollment

Enrollment	Total	Autism II
Families Enrolled	19	9
Subjects Enrolled	73	31
Families Concurrently Enrolled in CORA[a]	6	2

Note: Reflects ACTIVE families only.
[a]Only families enrolled through CORA are included in the statistics for Tables 1 and 2.

Human subjects and animal use protocols for this project are as follows:

- SGE-C Protocol #FSG20090011H – A Collaborative Translational Autism Research Program for the Military (NCH IRB #06-00360 and Wright-Patterson Medical Center (WPMC) IRB #FWP20060021H)
- SGE-C Protocol #FSG20120009H – Genomic Studies of Developmental and Cognitive Aspects of PTEN Disorders (PTEN) (NCH IRB #10-00138)
- SGE-C Protocol #FSG20120011H – OSU subcontract, Epigenetics of Drug Addiction Genes (Review by OSU IRB and AF Surgeon General's Human and Animal Research Protections Committee determined that this protocol is non-human subjects research and does not require further review, Feb 2013)
- SGE-C Protocol #AFDW-2013-001A – Functional Studies of Autism Candidate Genes in the Mouse (NCH IACUC #AR12-0072; Closed November 3, 2014) and Mouse Models of X-linked Developmental Disorders (NCH IACUC #04802AR)

On November 4, 2014, we were notified that USAF Headquarters had completed a review and would allow WPMC to rely upon the NCH IRB for the CORA project. WPMC could rely on the NCH IRB for regulatory oversight of the CORA protocol in accordance with DoD Instruction 3216.02, AF Instruction 40-402, and the Institutional Agreement for IRB Review for this study (executed July 2014). Copies of all NCH IRB actions pertaining to the CORA protocol were forwarded to WPMC for inclusion in a local "shadow" protocol file. Upon completion of Autism II, the protocol at WPMC will be closed and copies of NCH IRB actions pertaining to the CORA protocol, including yearly progress reports and any protocol amendments, will be forwarded directly to AF/SGE-C at usaf.pentagon.af-sg.mbx.afmsa-sge-c@mail.mil as long as Dr. Herman keeps the protocol active.

4.0 AIM 4.2 – IDENTIFY NEW AUTISM SUSCEPTIBILITY VARIANTS USING NGS TECHNOLOGY BY (a) DIRECT EXOME SEQUENCING OF SELECTED FAMILIES IN THE REGISTRY AND (b) ASSOCIATION STUDIES OF NOVEL REGULATORY AND GENE SPLICING VARIANTS THAT DEMONSTRATE AEI IN PREFRONTAL CORTEX BETWEEN AUTISM AND CONTROL BRAINS

4.1 Task 4.2.1 – Perform Exome Sequencing and Analyze the Data on 210 Individuals over the Course of this Project, Including 100 Individuals in Year 3 (Herman Lab)

As noted below, exome sequencing was performed on a total of 215 individuals in Autism II, including 103 in year 3 (Table 5). Given the advances in technology and bioinformatics for exome sequencing over the course of this project, as well as our understanding of the pathogenesis of ASDs, we modified our approaches accordingly. We concentrated our efforts in Autism II on families with a single child affected with ASD (sporadic cases) based on numerous reports in the literature beginning in ~2012 on the identification of pathogenic *de novo* variants in ~5% of sporadic cases [6-8,21,22]. In some of the studies, the rate of pathogenic *de novo* variants was higher in individuals with significant intellectual disability in addition to an ASD.

Table 5. Completed Exome Sequencing

Cohort	Total Autism I and II		Autism II Only	
	# Families	# Individuals	# Families	# Individuals
CNTN4	6	22	0	0
PTEN	16	48	14[a]	45
Simplex	75[b]	225	53	159
Multiplex	11	49	2	11

[a]Please note that 3 of the *PTEN* families were re-sequenced so that all sequencing results would be completed using the same exome platform. This will simplify data analysis and interpretation of the exome results.

[b]Results for 3 families (1 family each) were unable to be interpreted due to 1) identified nonpaternity via exome, 2) lack of primary DNA sample to confirm potential *de novo* WES findings, and 3) missing paternal DNA sample. In total 72 sporadic families were analyzed.

For exome sequencing, in Autism I, we initially employed proband DNA prepared from cell lines if sufficient primary DNA prepared directly from blood was not available. However, we noted a high *de novo* false positive rate, likely due to mutations occurring during establishment of the cell line. In Autism II, only primary DNA prepared directly from a blood sample was used for exome sequencing. Prior to exome sequencing, DNA samples were analyzed for quality as well as paternity, the latter using a SNP-Plex assay performed in the NCH High Throughput Genotyping Core. Exome sequencing, including capture using Agilent kits, was performed in the Biomedical Genomics Core under the direction of Dr. Peter White, PhD.

An in-house pipeline developed by Dr. White called "Churchill" [23] was used for data analysis. Stringent criteria were developed and applied as filters to the raw sequence data. These included the following:

1. At least 10X coverage for any variant for the proband and each parent in the trio sequenced (average coverage across all exomes was >60x with ~95% of targeted regions having at least 10x coverage)
2. A stringent quality score of ≥ 30 for each member of the trio calculated from the GATK variant caller in the Churchill pipeline
3. <2 alternate reads in either parent for the potential *de novo* variant identified in the proband
4. Variant resulted in predicted lack of the protein via nonsense or frameshift mutation or occurred in a canonical splice site
5. Missense variant predicted to be damaging by polyphen2 and/or SIFT [scale-invariant feature transform] protein prediction software
6. Variant was not found or was present at <1% frequency of the minor allele in available public databases (such as 1000 Genomes, ESP6500, Exac Browser, etc.)

Using the above criteria, more than 175 potential *de novo* variants were identified in probands from the 72 analyzed sporadic families. Validation was performed on 143 of the variants by Sanger sequencing on a sample prepared from primary DNA (blood or occasionally a cheek swab) (Table 6). Confirmation was not performed on potential *de novo* variants for genes unlikely to be causative (such as mucins, titin, etc.) or genes known not to be expressed in the central nervous system (CNS). Using primary DNA for exome sequencing in the proband, 85-90% of the potential *de novo* variants were validated, while only 25-30% were validated if exome sequencing was performed using cell line DNA.

Table 6. Validated Autism *de novo* Variants

Variants	N
Total De Novo Variants Analyzed	143
Confirmed in Primary	69/143
Failed to Validate	74/143

From the validated *de novo* variants identified, seven were considered pathogenic or likely pathogenic based on the predicted change in the protein and convincing reports in the literature demonstrating that the gene is involved in human ASD and/or intellectual disability (see Table 7). For five of the genes and families (*IQSEC2, CASK, SCN1A, AHDC1,* and *SRCAP*), results were communicated to the referring physician and families were seen by Dr. Herman for a clinical genetics appointment to discuss the findings and their implications, including low recurrence risk (likely <1%) for the parents or any close relatives. In addition, clinical confirmation in the NCH Molecular Genetics Laboratory (with charges) was recommended in all of these cases. A brief description of each gene is provided below:

IQSEC2 – X-linked gene that is a rare cause of intellectual disability in affected hemizygous males. They may also have ASD, seizures, and behavior/psychiatric problems. There are rare reports of intellectual disability and/or seizures in affected (carrier) females secondary to the pattern of X-inactivation. The IQSEC2 protein is expressed in CNS and is a guanine nucleotide exchange factor for the ADP-ribosylation factor (Arf) family of small GTPases, some of which have been associated with intellectual disability [24,25]. Our patient (female) has autism (ADOS 16 with cutoff of 10) and IQ of 64.

CASK – X-linked gene that encodes a calcium/calmodulin dependent serine protein kinase. Phenotypes associated with pathogenic *CASK* mutations include microcephaly with cerebellar and pontine hypoplasia and X-linked intellectual disability. Severe phenotypes are seen primarily in females, with presumed male lethality [26]. An association has not been found in individuals with a primary diagnosis of ASD. In our patient, her ADOS is 18 (cutoff 16) with IQ on Stanford-Binet of 40 and on the Leiter of 56. She has minimal speech. Her ASD is likely secondary to her intellectual disability and fits with the known spectrum of *CASK* phenotypes in heterozygous females.

SCN1A – Encodes an autosomal voltage gated sodium channel. *De novo* dominant mutations in this gene are associated with a spectrum of seizure disorders, including Dravet syndrome and, more recently, with a few cases of ASD [6,7]. The proband in our family has a history of febrile and non-febrile seizures in infancy, controlled with Depakote. His ADOS is 14 (cutoff 10) and IQ on the Stanford-Binet is 60.

AHDC1 – This gene encodes an AT-hook DNA binding protein that was recently described in association with a new syndrome called Xia-Gibbs [27]. Features include intellectual disability and mild dysmorphic features. Two individuals in the Simons Simplex Collection of individuals with ASD [28] have pathogenic mutations in this gene. Our patient is an 18-year-old male with significant intellectual disability who also carries a diagnosis of ASD.

SRCAP – Heterozygous *de novo* pathogenic (usually truncating) mutations in *SRCAP* have recently been associated with a rare genetic disorder called Floating-Harbor syndrome [29,30]. Features include short stature, intellectual disability, and distinctive facies. Mutations in the gene have also been reported in a few children with ASD. Our patient does not appear to have the syndrome, but does have ASD with an ADOS of 14 (cutoff 9) and full scale IQ of 62 (Stanford-Binet).

Two additional likely or possibly pathogenic variants were identified. However, given uncertainty of their association with disease, these have not been communicated to the involved families (see Table 7).

SYBU – *Sybu* (syntaxin-1 binding protein) interacts with the neural adhesion molecular syntaxin. It attaches syntaxin-containing vesicles in neurons to microtubules and is involved in their transport to synapses [31,32]. An unpublished knockout mouse model of the gene has behavior problems [Sheng ZH. Personal communication; 2014 Jul 25]. Since no human patients with intellectual disability and/or ASD have been described with mutations in this gene, we have not

reported the variant to the involved family. It remains a promising, albeit rare, ASD candidate gene.

KDM5B – This gene encodes a histone demethylase involved in epigenetic DNA modification. Specifically, it demethylates a lysine on histone H3 [33]. Its role in intellectual disability and ASD has been controversial, although two recent publications suggest that it may play a causative role in rare cases [21,22]. We will reevaluate whether to bring this family to clinic in the near future.

Table 7. Pathogenic and Likely Pathogenic Variants Identified by WES in ASD Families

Gene	Gene Name	DNA Variant	Predicted Protein Change	Coding Change	Inheritance	Family Notified
IQSEC2	IQ Motif- and SEC7 Domain-Containing Protein 2	c.1464dupT	p.E489_E490delinsX	Frameshift	De Novo	Yes
CASK[a]	Calcium/Calmodulin-Dependent Serine Protein Kinase	c.1052T>G	p.L351R	Missense	De Novo	Yes
SCN10A[a,b]	Sodium Channel, Voltage-Gated, Type X, Alpha Subunit	c.2306C>G	p.S769C	Missense	De Novo	Yes
SCN1A	Sodium Channel, Neuronal Type 1, Alpha Subunit	c.1147_1148insAATTT	p.F383_W384delinsX	Frameshift	De Novo	Yes
AHDC1	AT-Hook DNA-binding Motif-Containing Protein 1	c.1819G>A	p.D607N	Missense	De Novo	Yes
SYBU	Golgi-Localized Syntaphilin-Related Protein	c.176G>A	p.R59Q	Missense	De Novo	No
SRCAP	SNF2-Related CBP Activator Protein	c.431T>C	p.M144T	Missense	De Novo	Yes
KDM5B	Lysine-Specific Demethylase 5B	c.648delG	p.R216fs	Frameshift	De Novo	No
KCNQ3[b]	Potassium Channel, Voltage-Gated, KQT-Like Subfamily, Member 3	c.731G>A	p.R244H	Missense	AD, Maternal	Yes
FGD1[c]	FYVE, RhoGEF, and PH Domain-Containing Protein 1	c.935C>T	p.P312L	Missense	X-linked Recessive, Maternal	Yes

[a]The *CASK* and *SCN10A* variants were identified in the same individual.
[b]Secondary reportable variants unrelated to the ASD diagnosis in the family.
[c] Variant listed in ClinVar in 2002 as Pathogenic – Syndromic X-linked mental retardation 16 NM_004463.2(FGD1):c.935C>T (p.Pro312Leu).

We also identified two reportable secondary findings in distinct families sequenced. As defined by the American College of Medical Genetics and Genomics [34], these are known or predicted pathogenic variants in known disease-associated genes where recognition would result in prevention or significant amelioration of burdensome symptoms. They are:

SCN10A – This gene encodes a voltage-gated sodium channel associated with arrhythmias and sudden cardiac death (Brugada syndrome) [35]. The gene is on the American College of Medical Genetics and Genomics incidental finding list. This variant was identified in the same individual as the pathogenic *CASK* mutation.

KCNQ3 – This gene encodes a voltage-gated potassium channel and has been associated with benign familial neonatal seizures, as well as, more recently, non-febrile seizures, epilepsy, and intellectual disability [36]. Exome sequencing was performed in several members of a family with a son with ASD, but no seizures, and other family members (mother, two sisters, and one maternal half-sister) with neonatal and other types of seizures/epilepsy. The proband with ASD

did not carry the pathogenic familial variant, while all individuals with seizures did. One younger sibling was subsequently diagnosed with ASD, and it is not clear whether her *KCNQ3* mutation plays a role in her autism since her brother with ASD does not carry the variant. We consider this a reportable incidental or secondary finding at this point.

Finally, we identified a maternally inherited missense variant (Pro312Leu) in the X-linked *FGD1* gene. Mutations in this gene cause Aarskog syndrome, which had been considered previously in this child. Further, the variant we identified has been reported as pathogenic in one patient in the ClinVar database at the National Center for Biotechnology Information. The proband has hypertelorism and a shawl scrotum, which are key features of the disorder. His ADOS was 15 (cutoff 7) and his full-scale IQ was 52. Since this family no longer resides in Ohio, they were contacted by Mrs. Hansen-Kiss and referred for genetic counseling and clinical confirmation of the variant at a local institution.

We also performed exome sequencing on a total of 11 multiplex families with 2 or more affected siblings. Two of these families were sequenced as part of Autism II. No pathogenic or likely pathogenic variants in known or good candidate genes segregating with disease were identified in these families.

The exome sequencing results on the sporadic families will be made available for other researchers and incorporated into clinical care by physicians. We are preparing a manuscript for publication that should be submitted in the next 3-6 months. It will describe the exome sequencing results in sporadic families, including the pathogenic variants identified (Table 7). Other validated *de novo* variants will be listed as supplemental material. We will also share our data with Dr. Buxbaum and the ASC prior to publication, since they might have one or more individuals in their study with pathogenic variants in the same gene(s). Finally, as required for publication, raw exome sequencing data (BAM files) will be deposited in the dbGAP public database at the time of publication. These data become available to all researchers at that time.

4.2 PTEN Study

As noted above, exome sequencing was performed on a total of 14 families segregating a pathogenic *PTEN* mutation. In 9/16 families, the mutation was *de novo*. Validation was performed on a total of 27 potential *de novo* variants in the *PTEN*+ probands (excluding the *PTEN* gene itself) (Table 8). None were considered pathogenic or likely to influence/modify the phenotype in an affected. In collaboration with Dr. White's group, we continue to analyze inherited variants, particularly those in relevant pathways (PTEN, PI3K-AKT, WNT), to try to ascertain whether they could play a role in modifying the phenotype. In particular, we are trying to assess whether there is an excess of secondary damaging variants in probands with an ASD vs. those without ASD.

Table 8. Validated PTEN *de novo* Variants

Variants	N
Total PTEN *De Novo* Variants Analyzed	27
Confirmed in Primary	19/27
Failed to Validate	8/27

4.3 Task 4.2.2 – Perform Deep Transcriptome Sequencing and Analyze Data on 40 Brain Samples, 20 from ASD patients (+5 Replicates if Needed) over 3 Years (Sadee Lab)

In the past the Sadee laboratory has used sophisticated multiplex polymerase chain reaction (PCR) of RNA samples from a variety of tissues to examine expression differences in genes of interest depending on the specific disorder [37,38]. With the advent of NGS technologies, deep sequencing (>500x) of cDNAs can be employed to evaluate most mRNAs (the transcriptome) from a tissue in a single experiment, called RNA-seq [39]. For this project, the Sadee lab prepared 40 samples for RNA-seq from ASD and unaffected cerebral cortex. All 40 samples were prepared at the same time and handled the same way to avoid batch effects generated by running samples at different times on different platforms. Suitable libraries could not be generated from nine of the samples, even after repeated attempts to re-isolate RNA from the original tissue samples, presumably due to poor quality/degradation of the sample and/or low concentration of the resulting RNA. We were able to generate usable transcriptome sequence data from 31 samples. Sequencing reads from the Ion Proton run were mapped to a reference sequence using Tophat and Bowtie software (Figure 1). Various quality filters were applied and then edgeR was used for differential expression analysis between cases and controls. A gene ontology term query revealed enrichment for immune-related genes, supportive of similar results in the literature [40] (Figure 2). We also have measured AEI in these samples and identified several additional genes that show the presence of cis-acting variants. We have also obtained whole genome SNP chip analyses for comparison to the RNA-seq data, which allows us to search for functional regulatory variants on a large scale. The analyses are continuing with a focus on ASD related genes.

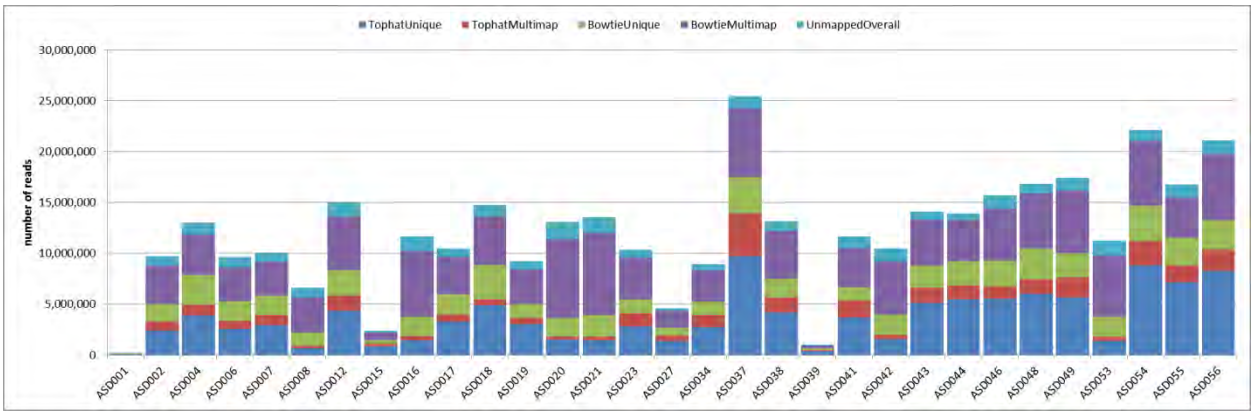

Figure 1. Transcriptome sequence data for each sample. Sequencing reads from the Ion Proton run were mapped to a reference sequence using Tophat and Bowtie software.

1: GO: Molecular Function [Display Chart] 630 annotations before applied cutoff / 18326 genes in category

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	GO:0003823	antigen binding		9.590E-10	6.042E-7	4.244E-6	6.042E-7	13	101
2	GO:0019864	IgG binding		4.755E-9	1.498E-6	1.052E-5	2.996E-6	6	12
3	GO:0032403	protein complex binding		8.403E-9	1.765E-6	1.239E-5	5.294E-6	36	924
4	GO:0005125	cytokine activity		3.139E-8	4.408E-6	3.096E-5	1.978E-5	16	213
5	GO:0005102	receptor binding		3.735E-8	4.408E-6	3.096E-5	2.353E-5	45	1405

Show 40 more annotations

2: GO: Biological Process [Display Chart] 4095 annotations before applied cutoff / 18482 genes in category

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	GO:0002682	regulation of immune system process		3.508E-34	1.167E-30	1.038E-29	1.437E-30	80	1212
2	GO:0002684	positive regulation of immune system process		5.697E-34	1.167E-30	1.038E-29	2.333E-30	64	732
3	GO:0006952	defense response		1.103E-33	1.505E-30	1.339E-29	4.516E-30	88	1515
4	GO:0006955	immune response		4.793E-31	4.907E-28	4.365E-27	1.963E-27	82	1416
5	GO:0009611	response to wounding		1.202E-30	9.847E-28	8.759E-27	4.924E-27	77	1255

Show 45 more annotations

3: GO: Cellular Component [Display Chart] 370 annotations before applied cutoff / 18865 genes in category

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	GO:0005615	extracellular space		2.785E-26	1.031E-23	6.690E-23	1.031E-23	72	1284
2	GO:0072562	blood microparticle		1.582E-20	2.927E-18	1.900E-17	5.855E-18	24	132
3	GO:0016023	cytoplasmic membrane-bounded vesicle		3.335E-9	4.067E-7	2.640E-6	1.234E-6	40	1062
4	GO:0031410	cytoplasmic vesicle		4.397E-9	4.067E-7	2.640E-6	1.627E-6	42	1163
5	GO:0009986	cell surface		6.045E-9	4.474E-7	2.904E-6	2.237E-6	31	700

Show 45 more annotations

4: Human Phenotype [Display Chart] 2071 annotations before applied cutoff / 4141 genes in category

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	HP:0010987	Abnormality of cellular immune system		9.873E-10	1.022E-6	8.397E-6	2.045E-6	26	386
2	HP:0001881	Abnormality of leukocytes		9.873E-10	1.022E-6	8.397E-6	2.045E-6	26	386
3	HP:0001871	Abnormality of blood and blood-forming tissues		2.704E-9	1.867E-6	1.533E-5	5.601E-6	41	960
4	HP:0002715	Abnormality of the immune system		2.017E-8	1.044E-5	8.579E-5	4.178E-5	39	936
5	HP:0010978	Abnormality of immune system physiology		5.403E-7	2.238E-4	1.838E-3	1.119E-3	28	594

Show 45 more annotations

5: Mouse Phenotype [Display Chart] 3217 annotations before applied cutoff / 7978 genes in category

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	MP:0001545	abnormal hematopoietic system physiology		2.913E-25	9.371E-22	8.110E-21	9.371E-22	89	1399
2	MP:0012382	abnormal blood cell physiology		7.616E-25	1.225E-21	1.060E-20	2.450E-21	86	1323
3	MP:0001819	abnormal immune cell physiology		1.914E-24	2.048E-21	1.772E-20	6.157E-21	83	1248
4	MP:0002442	abnormal leukocyte physiology		2.547E-24	2.048E-21	1.772E-20	8.193E-21	82	1223
5	MP:0002421	abnormal cell-mediated immunity		3.336E-24	2.146E-21	1.857E-20	1.073E-20	83	1258

Show 45 more annotations

Figure 2. Gene ontology term query. Read counts were filtered and required to have average expression > 1 CPM in one category (ASD or control). Counts were then used for differential expression analysis in edgeR. Genes with a false discovery rate (FDR) less than 0.05 were included in gene ontology (GO) term enrichment demonstrating a link with immune function.

4.4 Task 4.2.3 – Identify Functional Variants for Up to 5 Newly Identified Candidate Genes with Frequent AEI (Identified in Task 4.2.2) (Sadee Lab)

In addition to the RNA-seq analyses, we remain focused on resolving functional genetic variants for relevant disease-related genes. We had previously identified rs11633223 as a candidate SNP in the acetylcholine receptor subunit gene *CHRNA3* (Figure 3). We confirmed an association with mRNA expression using qualitative PCR and the transcriptome data. There are several blocks of variants in high linkage disequilibrium across the α5-α3-β4 gene cluster. Therefore, to isolate the effect of the specific SNP rs11633223, we created a gene construct with either the major or minor allele attached to a luciferase reporter and demonstrated an effect on protein expression in this assay (Figure 4). We can now begin to study the interactions between three functional variants in the α5-α3-β4 gene cluster, which consists of a large haplotype block, suggesting functional connectivity between genes. Such interactions have yet to be tested in clinical association studies and may be relevant to ASD.

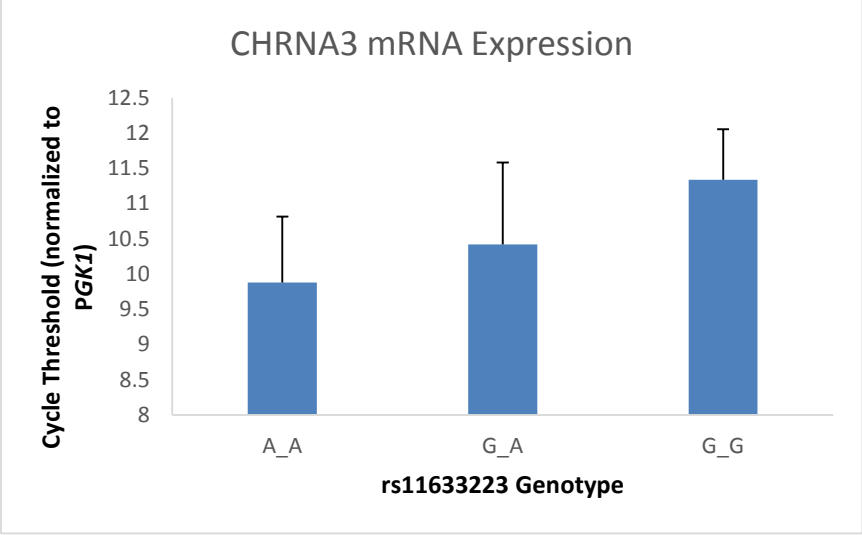

Figure 3. Protein expression of *CHRNA3* rs11633223. *CHRNA3* total mRNA expression measured in post-mortem brain, standardized with *PGK1* and stratified by rs11633223 genotype. The quantitative real time PCR cycle thresholds are standardized to the house keeping gene; a higher cycle threshold denotes lower expression.

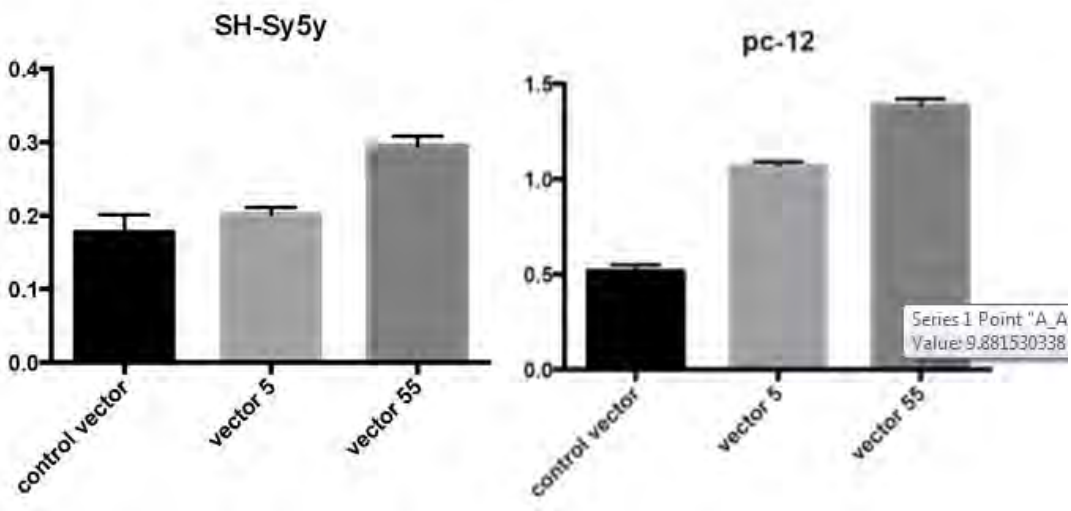

Figure 4. Luciferase assay of *CHRNA3* fragment in SH-SY5Y and PC-12 cells. The minor C allele of rs11633223 (vector 55) showed increased activity compared to the major T allele (vector 5) and the control vector.

As we have previously described, the glial glutamate transporter gene *SLC1A3* likely contains a regulatory genetic variant, based on evidence from our RNA-seq data. We scanned all variants 500,000 bases in either direction up or downstream from the gene, and the SNP rs17358298 emerged as the strongest candidate, with the highest association between AEI and genotype. This SNP was then genotyped in the CORA cohort. However, we did not find an association between genotype and case status.

Several additional regulatory variants in candidates identified in the Sadee lab or literature were examined in the CORA cohort as discussed below.

4.5 Task 4.2.4 – Perform Association Studies for Relevant Candidate Genes with Frequent AEI Identified in Up to 200 Trios in CORA and Analyze Resulting Data (Identified in Task 4.2.3) (Herman Lab, Sadee Lab)

4.5.1 Analyses of Regulatory, Non-Coding SNPs Relevant to ASD. The Herman and Sadee labs examined 14 SNPs exhibiting significant AEI that were discovered and/or studied in the Sadee lab in approximately 200 trios in CORA (proband plus parents). Significant transmission distortion was found for a single SNP rs6311 in the serotonin receptor 2A gene, as detailed in our published manuscript [41]. No additional individual SNPs beyond rs6311 (HTR2A) were found to be associated with autism (Table 9). More recently, we tested for SNP x SNP interactions, an approach made feasible by careful selection of only a few frequent causative variants in strong candidate genes. The top results are shown in Table 10, suggesting significant interactions between neurotransmitter genes with substantial effect size. These promising leads need to be replicated in larger cohorts. To this end, Dr. Sadee applied for and gained access to two autism Genome-Wide Association Studies datasets to be used for validation: Autism Genetic Resource Exchange and Simons Foundation Autism Research Initiative (SFARI). His lab is currently imputing our SNPs of interest that are missing from the SFARI dataset. Then they can test epistatic interactions between their functional SNPs and determine whether the results from CORA replicate in larger cohorts.

Table 9. Single SNP Analysis of all SNPs Genotyped in the CORA Cohort

CHR	SNP	Gene	BP	Freq Case	Freq Ctrl	Transmitted	Untransmitted	OR	CHISQ	P
9	rs1108580	DBH	136505114	0.46	0.49	96	116	0.83	1.89	0.17
15	rs11633223	CHRNB4	78935476	0.34	0.35	90	98	0.92	0.34	0.56
11	rs12364283	DRD2	113346955	0.06	0.07	21	27	0.78	0.75	0.39
9	rs12682807	SLC1A1	4574022	0.11	0.10	31	29	1.07	0.07	0.80
9	rs1611115	DBH	136500515	0.21	0.22	68	75	0.91	0.34	0.56
15	rs16969968	CHRNA5	78882925	0.36	0.36	63	65	0.97	0.03	0.86
5	rs17358298	SLC1A3	36445802	0.13	0.14	44	54	0.81	1.02	0.31
23	rs2064070	MAOA	43608682	0.30	0.29	26	30	0.87	0.29	0.59
6	rs2144025	ESR1	152307706	0.18	0.18	57	61	0.93	0.14	0.71
11	rs2283265	DRD2	113285536	0.17	0.16	56	54	1.04	0.04	0.85
5	rs27072	DAT_ SLC6A3	1394522	0.15	0.16	55	61	0.90	0.31	0.58
13	rs6311	HTR2A	47471478	0.38	0.42	79	117	0.68	7.37	**0.01**
13	rs6314	HTR2A	47409034	0.12	0.11	32	27	1.19	0.42	0.52
5	rs6347	DAT_ SLC6A3	1411412	0.27	0.26	79	66	1.20	1.17	0.28
12	rs7305115	TPH2	72372862	0.42	0.41	110	97	1.13	0.82	0.37
15	rs880395	CHRNA5	78844356	0.35	0.36	77	82	0.94	0.16	0.69

Note: BP = base pair; CHISQ = chi square; CHR = chromosone; OR = odds ratio.

Table 10. Top SNP x SNP Interactions with an Additive or Dominant Model

SNP1	Gene1	SNP2	Gene2	Additive Model							Dominant Model						
				Coef	RR	Lower	Upper	SE	Stat	p-value Full Add	Coef	RR	Lower	Upper	SE	Stat	p-value Full D
rs17358298	SLC1A3	rs1611115	DBH	0.678	**1.97**	1.16	3.35	0.27	6.27	0.012	0.92	**2.50**	1.17	5.33	0.39	5.65	0.018
rs6311	HTR2A	rs6347	DAT_SLC6A3	-0.440	**0.64**	0.44	0.93	0.19	5.38	0.020	-0.15	**0.86**	0.45	1.65	0.33	0.20	0.657
rs880395	CHRNA5	rs12682807	SLC1A1	-0.686	**0.50**	0.27	0.93	0.31	4.79	0.029	-0.91	**0.40**	0.16	0.99	0.46	3.93	0.047
rs11633223	CHRNB4	rs12682807	SLC1A1	-0.598	**0.55**	0.30	1.01	0.31	3.72	0.054	-0.66	**0.52**	0.21	1.28	0.46	2.04	0.153
rs11633223	CHRNB4	rs27072	DAT_SLC6A3	-0.473	**0.62**	0.39	1.01	0.25	3.70	0.054	-0.80	**0.45**	0.22	0.91	0.36	4.98	0.026
rs11633223	CHRNB4	rs2283265	DRD2	0.423	**1.53**	0.94	2.48	0.25	2.90	0.088	0.76	**2.15**	1.06	4.35	0.36	4.49	0.034
rs6347	DAT_SLC6A3	rs880395	CHRNA5	-0.209	**0.81**	0.54	1.21	0.20	1.04	0.307	-0.88	**0.42**	0.20	0.86	0.37	5.54	0.019
rs6347	DAT_SLC6A3	rs7305115	TPH2	0.074	**1.08**	0.75	1.54	0.18	0.17	0.685	-0.20	**0.82**	0.42	1.58	0.34	0.36	0.549

Note: SNPs in the same gene or in high linkage disequilibrium are not included in the analysis as these violate the assumptions of the model.
RR = relative risk; SE = standard error; Stat = statistic.

4.5.2 Analysis of CNVs in Immune-Related Genes. To test the hypothesis that variation in the strength of the immune system can contribute to genetic risk for ASD [40], in collaboration with Dr. Chack Yung Yu, Professor in Molecular and Human Genetics, NCH Research Institute, we investigated the multi-allelic and frequent CNVs for two sets of innate immunity genes: complement C4A and C4B from chromosome 6p21.3 and neutrophil alpha-defensins DEFA3 and DEFA1 from chromosome 8p23.1. Complement proteins are important humoral immune effectors that neutralize infectivity or form pores on microbes. Defensins are cationic antimicrobial peptides. In addition to a role in defense against infections during fetal development, complement proteins have been shown to be important in neurodevelopment, where they are involved in synapse rewiring and pruning.

Our study population included 166 ASD patients and 500 healthy controls of European ancestry. The gene copy number of C4A varied from 0 to 5. When compared with controls, we observed a significant increase in the frequency of complement C4A deficiency in ASD patients [C4A gene copy number = 0 or 1, OR=1.52 (1.02-2.27), p=0.029]. The gene copy numbers of DEFA3/A1 ranged from 4 to 23. The lower copy number group with 3 and 8 copies of

DEFA3/A1 genes had significantly higher frequency in ASD when compared with controls. DEFA3 has the A65D mutation that disrupts charge (less basic) and may not be advantageous functionally. A complete absence of DEFA3 was present in 10.8% of ASD but 18.1% in healthy subjects, suggesting that the absence of DEFA3 might be protective against ASD with an OR=0.55 (0.31-0.98), p=0.035.

These preliminary studies suggest that common CNVs of innate immune response genes for complement and defensins may be medium effect size risk factors for ASD. Future plans in the Yu lab include extending the study to include mothers of ASD patients to investigate if maternal defense during pregnancy might affect brain development. In addition, the studies need to be replicated in larger ASD study populations to firmly establish if common CNVs in these, and perhaps other, immune defense genes contribute to genetic risks for ASD.

4.6 Task 4.2.5 – Perform Sanger Sequencing for Up to Five Novel Genes with Pathogenic Variants Identified by Exome Sequencing in Entire CORA Cohort (Identified in Task 4.2.1) (Herman Lab)

No suitable novel candidate genes were identified for Sanger sequencing in the CORA cohort.

5.0 AIM 4.3 – DEMONSTRATE THE SIGNIFICANCE OF THE X-LINKED CANDIDATE AUTISM GENE *Nsdhl* BY PERFORMING PRELIMINARY FUNCTIONAL STUDIES USING A LIVE MODEL

5.1 Task 4.3.1 – Select First Gene for Functional Studies

The generation of animal models (mouse, zebrafish, etc.) is probably one of the best approaches to demonstrate the significance of a candidate gene or specific mutation and to begin to elucidate its function in the brain and its role in behavior. Dr. Herman obtained permission in August 2013 to analyze behavior in a conditional targeted allele of the X-linked *Nsdhl* gene. *Nsdhl* encodes an enzyme involved in one of the later steps of cholesterol synthesis. The Herman lab has studied the gene for many years and had recently generated and validated a conditional allele that can be employed to inactivate the gene in specific tissues and at specific times of interest (called *Nsdhl* CKO [male conditional knockout]) [42].

5.2 Task 4.3.2 – Characterize and Perform Functional Studies of Targeted Gene 1 (*Nsdhl*) (Identified in Task 4.3.1)

Consequences of inactivation of *Nsdhl* in the CNS depend on the time and cell type involved, as well as on the robustness of the Cre line used. In our published work, prenatal inactivation of the gene using *GFAP-cre* that is expressed beginning at embryonic day 13 in most differentiating neurons and glia astrocytes resulted in death by postnatal day (P) 15-20. To try to examine behavioral phenotypes in adult animals, we inactivated the gene in post-mitotic neurons at ~P20 using a constitutive Camk2a-Cre mouse line. This Cre line provides robust neuronal loss (>95%). However, the resultant mice were healthy, fertile, and had no observable phenotype. Histology and immunostaining for NSDHL protein demonstrated efficient gene inactivation.

For the studies here, we sought to inactivate *Nsdhl* in mature cortical astrocytes using a tamoxifen-inducible *Fgfr3-iCreERT2*. As shown in Table 11, activation of Cre using a tamoxifen dose of 200 µg/g body weight at ~P45 leads to wasting and death of the mutant mice (sacrificed due to greater than 10% loss of body weight). We believe the weight loss results from *Nsdhl* inactivation and cell death in the hypothalamus, a region of the brain concerned with hunger and satiety. Using lower doses of tamoxifen in adult mice, we were able to generate a cohort of animals that could be used for behavior studies. In comparison with wild type male littermates (WT), mutant males in cohorts 2 and 3 demonstrated increased grooming, which is correlated with anxiety and, perhaps, obsessive-compulsive behavior (Table 11). The mutant mice in all three cohorts that underwent behavioral testing (cohorts 2-4) were also less aggressive in the tube test for social dominance. In cohorts 2 and 4, there was decreased activity in the open field, which may also signify anxiety, while increased activity on the running wheel over 36 hours could indicate hyperactivity. One confounding factor for these results could be the influence of decreased weight of the mutant mice compared with wild type littermates.

Table 11. Summary of Behavior Testing Performed on Male Offspring of *Fgfr3-cre* x *Nsdhl* CKO Mice

Item	Cohort 1	Cohort 2	Cohort 3	Cohort 4
Tamoxifen dose (no. x µg/g body weight)	3 x 200	3 x 100	3 x 100	3 x 150
Age 1st dose of tamoxifen administered	P45	P45	P45	P42
Number of animals	3 WT, 5 CKO[a]	8 WT, 7 CKO	6 WT, 9 CKO	22 WT, 19 CKO
Behavior tests[b]				
Grooming	ND	increased	increased	no difference
Tube test of social dominance	ND	decreased	decreased	decreased
Elevated plus maze	ND	no difference	no difference	no difference
Open field	ND	decreased activity	no difference	decreased activity
Morris water maze	ND	delayed learning	no difference	no difference
3-chamber sociability	ND	no difference	no difference	ND
Acoustic startle prepulse inhibition	ND	decreased	no difference	no difference
Running wheel activity	ND	increased	no difference	no difference

Note: ND = not determined.
[a]CKO males were euthanized due to weight loss >10% body weight.
[b]All differences listed for specific behaviors tests (i.e., increased, decreased, delayed learning) between CKO and WT males were significant $p \leq 0.05$.

In a preliminary experiment, we demonstrated inactivation of *Nsdhl* in *Fgfr3-Cre* expressing cells by double-label immunofluorescence with antibodies to the NSDHL protein and S100B, a robust protein marker for this subpopulation of cortical astrocytes. Counting 10 fields from the cerebral cortex from 2 mice of each genotype sacrificed 16 days after the last dose of tamoxifen, *Nsdhl* was expressed in 77± 3.1% of S100B+ cells in wild type cortex, but was expressed in only 27±13% of mutant S100B+ astrocytes. Additional wild type and mutant brains will be analyzed, as well as examining additional regions of the brain such as the hippocampus, hypothalamus, and cerebellar cortex. No obvious cell death was noted, although TUNEL [terminal deoxynucleotidyl transferase dUTP nick end labeling] analysis for apoptosis has not as yet been performed.

These preliminary studies demonstrate inactivation of *Nsdhl* in a subpopulation of mature cortical astrocytes is associated with behavioral phenotypes. They are consistent with the belief that cholesterol synthesis in the cortex of adult mice occurs in astrocytes with subsequent secretion and uptake by neurons [43]. Future studies will examine further consequences of the inability to synthesize cholesterol in these astrocytes, including whether neuronal cholesterol synthesis is upregulated. We will try to ascertain the mechanisms involved in these metabolic shifts.

6.0 PUBLICATIONS, ABSTRACTS/POSTERS, AND PRESENTATIONS

6.1 Publications

Smith RM, Banks W, Hansen E, Sadee W, Herman GE. Family-based clinical associations and functional characterization of the serotonin 2A receptor gene (*HTR2A*) in autism spectrum disorder. Autism Res. 2014; 7(4):459-467.

Smith RM, Webb A, Papp AC, Newman LC, Handelman SK, et al. Whole transcriptome RNA-Seq allelic expression in human brain. BMC Genomics. 2013; 14:571.

6.2 Abstracts and Posters

Banks W, Cunningham D, Hansen E, Ratliff-Schaub K, Butter E, et al. Exome sequencing of 43 sporadic cases with an autism spectrum disorder in a local cohort of families identifies severe de novo variants and implicates additional genes in ASD pathogenesis. Poster presented at the American Society of Human Genetics Annual Conference; 2014 Oct 18-22; San Diego.

Wang H, Lintner K, Hansen E, Zhou B, Wu YL, et al. DEFA3/A1 and complement C4A are medium effect-size risk factors for human autism spectrum disorders (ASD). Poster presented at the American Society of Human Genetics Annual Conference; 2013 Oct 23-26; Boston.

6.3 Presentations

Smith R. Identifying common functional regulatory poymorphisms in autism spectrum disorders using RNA AmpliSeq. Presentation to the Society of Neuroscience Annual Conference; 2013 Nov 9-13; San Diego.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADOS	Autism Diagnostic Observation Schedule
AEI	allelic expression imbalance
ASC	Autism Sequencing Consortium
ASD	autism spectrum disorder
BAP	broader autism phenotype
CKO	male conditional knockout mouse
CMA	chromosome microarray analysis
CNS	central nervous system
CNV	copy number variation
CORA	Central Ohio Registry for Autism
DNA	deoxyribonucleic acid
DoD	Department of Defense
IACUC	Institutional Animal Care and Use Committee
IQ	intelligence quotient
IRB	Institutional Review Board
NCH	Nationwide Children's Hospital
NGS	next generation sequencing
OR	odds ratio
OSU	The Ohio State University
P	postnatal day
PCR	polymerase chain reaction
PQ	pregnancy questionnaire
PTEN	phosphatase and tensin homologue deleted on chromosome ten
RNA	ribonucleic acid
SFARI	Simons Foundation Autism Research Initiative
SGE-C	Air Force Medical Support Agency Research Oversight and Compliance Division
SNP	single nucleotide polymorphism
SRS	Social Responsiveness Scale
WES	whole-exome sequencing
WPAFB	Wright-Patterson Air Force Base
WPMC	Wright-Patterson Medical Center
WT	wild type male littermate mouse